REMARKS

I. INTRODUCTION

In response to the Office Action dated August 25, 2003, claims 31, 36-39 and 44-47 have been amended. Claim 33 has been cancelled. Claims 40-43 have been withdrawn from consideration by the Examiner. Claims 31, 32, 34-39 and 44-47 remain in the application and are presently being examined. Entry of these amendments, and reconsideration of the application, as amended, is requested.

II. CLAIM AMENDMENTS

Applicants' attorney has made amendments to the claims as indicated above. These amendments were made solely for the purpose of clarifying the language of the claims, and do not introduce new matter. Support for the amendment to claim 31 can be found in cancelled claim 33. Support for the amendment to claims 44-47 can be found in the specification at page 9, line 11, and at page 11, lines 1 and 7. Entry of these amendments is respectfully requested.

III. INTERVIEW SUMMARY

Applicants' undersigned attorney acknowledges and appreciates the helpful comments and suggestions offered by Examiner Mosher during telephonic interviews conducted on October 7, 2003, and on February 11, 2004. The discussion included the definition of "synthetic peptide" and potential strategies for overcoming prior art rejections. Applicants have amended the claims and addressed outstanding issues in the arguments hereinbelow, in a manner consistent with Applicants' understanding of the Examiner's comments. Should the Examiner find that further issues remain that would interfere with allowance of the pending claims, the courtesy of a telephone call would be greatly appreciated.

In addition, the Examiner noted an error in the Examples portion of the specification. Applicants appreciate the Examiner's helpfulness in pointing out this error and the obvious correction. Accordingly, the specification has been amended at page 14 to correct the reference to the figures discussed therein.

IV. RESTRICTION REQUIREMENT

At page (2) of the Office Action, newly submitted claims 40-43 were withdrawn from consideration as being directed to a non-elected invention. Because all of the claims are linked by a common inventive concept, the provision of a method of high yield synthesis of Vpr that results in previously unattained synthetic Vpr proteins that are stable and soluble in aqueous solution, Applicants respectfully request the Examiner reconsider and withdraw the restriction requirement. Applicants note that the product claims have been amended to clarify their relationship to the method claims, making more explicit the common inventive concept that links the subject matter of all of the pending claims.

V. NON-ART REJECTIONS

A. Indefiniteness

At page (2) of the Office Action, claims 33, 34, 36, 38, and 44-47 were rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention.

The cancellation of claim 33 renders the rejection of this claim moot. The amendments to claim 31 clarify that "comprising" applies to all or a fragment or variant of SEQ ID NO: 1, and that sub-parts (b) and (c) refer to SEQ ID NOS: 2 and 3, respectively. The amendment of claims 44-47 to recite "product" instead of "system" removes any potential uncertainty that a composition of matter is intended, which is consistent with the recitation of physical components ("peptide" and "substrate"), rather than method steps, in the claims. Claims 46 and 47 have been further amended to clarify that this embodiment of the product of claims 44 and 45 is one in which the substrate referred to in the corresponding parent claims comprises an ELISA carrier surface.

B. Written Description

At page (3) of the Office Action, claims 34, 35, 38, 39, and 44-47 were rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Although Applicants pointed to portions of the specification which support these claims, these references to

support in the specification are dismissed as they allegedly "do not reasonably convey the broad concept of 'bound to a second molecule comprising a DNA or protein molecule'". Applicants respectfully disagree with this assertion.

It is well known in the art (as it was at the time the present application was filed) that Vpr is particularly suited for conjugation to other proteins or DNA. In addition, the specification refers to the "broad concept" in its discussion of using synthetic Vpr for gene transfer (see, e.g., page 12, lines 18-22). Moreover, the usefulness of Vpr molecules in fusion proteins (see, e.g., page 5, line 6) and otherwise bound to a second molecule (see, e.g., page 2, lines 27-28) is so widely known, the broad concept is readily recognized by those skilled in the art regardless of the recitation of specific examples. Even if one construes the recitation of ELISA applications as solely supporting immobilized antibody (and not the antigen bound to a second molecule), the reference to using Vpr or fragments thereof to generate antibodies (see, e.g., page 10, lines 22-23) also conveys the broad concept of Vpr bound to a second molecule, as fusion proteins are often used in the art for the generation of antibodies.

The assertion that the discussion in the specification (at page 10, line 27, and page 11, lines 13-15) of using the synthetic Vpr peptide of the invention in an ELISA "does not reasonably convey the concept of immobilized antigen". Applicants are puzzled by this assertion, as the specification explicitly recites this use in an ELISA as a use for the peptide (not the antibody recognizing the peptide). Moreover, those skilled in the art recognize ELISA to refer to assays that use both immobilized antigen and immobilized antibody, as evidenced by the entry found in the 1997 edition of the Oxford Dictionary of Biochemistry and Molecular Biology (see copy of relevant page in Exhibit A, submitted herewith). The entry circled at page 210 of the enclosed portion of this dictionary reference defines ELISA as "a form of quantitative immunoassay based on the use of antibodies (or antigens) that are linked to an insoluble carrier surface, which is then used to 'capture' the relevant antigen (or antibody) in the test solution."

The Examiner is respectfully reminded that the written description requirement does not necessitate recitation of the language *in ipsis verbis* (*In re Lukach*, 169 USPQ 795, 798, CCPA 1971). Compliance with 35 U.S.C. §112, first paragraph, "does not necessarily require specific recitations of use but may be inherent in description or may result from disclosure of a sufficient number of properties to make a use obvious; and where those of ordinary skill in the art will know how to use,

the applicant has a right to rely on such knowledge". (*In re Nelson*, 126 USPQ 242, 253, CCPA 1960). Applicants maintain that one skilled in the art would readily appreciate a description of Vpr peptides bound to DNA or protein molecules from the specification as filed.

C. Enablement

At page (4) of the Office Action, claims 36-39 were rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such as way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. This rejection is based on an assertion that the specification does not teach any treatment method of using the claimed peptides. Applicants respectfully disagree, as the specification recites a number of therapeutic uses for the claimed peptides, e.g., at page 10, line 26, and at page 12, as well as original claims 28-30. In addition, the Examiner alleges that "a search of the prior art does not indicate routine knowledge of successful treatment methods using HIV VPR peptides." Yet the prior art cited by the Examiner discloses several treatment methods using HIV VPR peptides (see, e.g., Abstracts of Azad and Weiner). In fact, the Examiner has even stated at page 6 of the Office Action, that it would have been obvious to prepare a pharmaceutical composition containing Vpr protein for therapeutic use. To facilitate prosecution, however, Applicants have amended claim 36-39 to delete "pharmaceutical" and "pharmaceutically acceptable".

At page (5) of the Office Action, claims 31, 34, 36, 38, 44, and 46 were rejected under 35 U.S.C. §112, first paragraph, because the specification, while being enabling for the recited VPR fragments, does not reasonably provide enablement for variant peptides SEQ ID NO:8 and 9. The Examiner alleges that "the specification does not suggest how to use variant peptides which do not match the sequence of VPR from any known strain of HIV". Applicants respectfully disagree with this statement.

The specification, at page 6, lines 20-21, discloses the use of the Vpr peptides of the invention for epitope mapping and isoelectric focusing. At pages 27-30 of the application, namely originally-filed claims 9-30, numerous uses for Vpr peptides having mutations that substitute the native prolines (as do SEQ ID NO: 8 and 9) are described. In addition to the uses discussed in the specification, additional evidence in the record supports uses appreciated by those skilled in the art. For example, the utility of substitution for these prolines is discussed in material that can be found

in the record at pages 6-8 (Example 19) of the Preliminary Amendment submitted with the application as filed on August 20, 2001. In particular, the Examiner's attention is directed to page 8, lines 1-7, of this Amendment. As discussed therein, those skilled in the art recognize that the substitution of asparagine for proline would allow for a similar effect (to proline) on the protein backbone, but without being able to undergo cis/trans isomerism, rendering them ideal for structural analyses using NMR and X-ray crystallography. These stable forms of the Vpr peptides are also useful for functional studies, for example, of ion channel activity. It is known in the art that ion channel function is located in the N-terminus of Vpr, and tools and molecules are necessary for these functional studies. Epitope mapping is also a utility that is relevant and substantial. Because the usefulness of Vpr is already appreciated, this is not the same as a research utility wherein the research is to identify what an unknown molecule can be used for. Research use is not, *per se*, an insubstantial utility, particularly where the value of the outcome of the research is already known. Rather, the present situation is analogous to an animal model that is useful in research relating to a known disease. Stable fragments of a protein having a known value are likewise useful in research directed at taking advantage of the known functions of the protein.

Accordingly, Applicants respectfully request the Examiner reconsider and withdraw the rejections based on 35 U.S.C. §112.

VI. PRIOR ART REJECTIONS

At page (5) of the Office Action, claims 31, 32, and 36 were rejected under 35 U.S.C. §102(b) as being anticipated by Azad, WO 95/26361 (Azad). Also at page (5) of the Office Action, claim 31 was rejected under 35 U.S.C. §102(b) as being anticipated by Sette et al., WO 98/32456 (Sette). At page (6) of the Office Action, claims 31, 34, and 44 were rejected under 35 U.S.C. §102(b) as being anticipated by Koprowski et al., WO 98/08375 (Koprowski). At page (6) of the Office Action claims 35 and 37 were rejected under 35 U.S.C. §103(a) as being unpatentable over Azad. At page (7) of the Office Action, claims 33, 34, 37, and 38 were rejected under 35 U.S.C. §103(a) as being unpatentable over Sette. At page (7) of the Office Action, claims 31-39 were rejected under 35 U.S.C. §103(a) as being unpatentable over Weiner et al., WO 96/08970 (Weiner).

Applicants respectfully traverse these rejections.

None of the cited references discloses a synthetic Vpr protein having the amino acid sequence shown in SEQ ID NO: 1. Applicants respectfully note that "synthetic" is defined in the specification at page 9, lines 22-25, as referring to "solid phase peptide synthesis". As discussed at page 9, lines 10-14, the ability to successfully produce a synthetic Vpr was surprising and unexpected given the results of previous attempts to provide a useful Vpr protein via synthetic or recombinant methods. The synthetic Vpr proteins of the invention, even at mM concentrations, are very soluble in water and remain stable without any sign of protein aggregation and protein precipitation.

In contrast, the Vpr proteins described in the cited references are made by yeast cells (Azad) or plant cells (Koprowski), and not via solid phase peptide synthesis. Moreover, the fragments described in the cited references are not encompassed by Applicants' claims. The Vpr molecules made by the prior art and the Vpr molecules made by solid phase synthesis are not the same. Vpr contains 11 residues ($Ser^{28, 79, 94, 96}$, $Thr^{19, 49, 53, 84}$, $Tyr^{15, 47, 50}$) capable of being phosphorylated and, as shown in the Journal of Virology article by Muller et al. submitted herewith as Exhibit B, Vpr is phosphorylated in cells. One or more of these phosphorylation sites is present in each of the synthetic Vpr peptides encompassed by Applicants' claims. Accordingly, even if the cited references pertaining to recombinantly produced Vpr were regarded as teaching or suggesting the molecules claimed by Applicants, these molecules are structurally distinct.

As discussed above, the successful synthesis of Vpr without problems involving protein aggregation and solubility in aqueous solution was unexpected. Thus, the various compositions and uses recited in claims 31-39 cannot be regarded as obvious, as the problems discussed in the review of the prior art at pages 2-5 of Applicants' specification would have prevented them from being enabled. The ability to prepare and use compositions comprising synthetic Vpr is not taught or suggested in the Azad, Weiner, Sette, or Koprowski references cited by the Examiner. Accordingly, Applicants respectfully request the Examiner reconsider and withdraw the rejections based on the prior art.

VII. CONCLUSION

In view of the above, it is submitted that this application is now in good order for allowance and such allowance is respectfully solicited. Should the Examiner believe minor matters still remain

that can be resolved in a telephone interview, the Examiner is urged to call Applicants' undersigned attorney.

Respectfully submitted,

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lyses the phosphorylation by phosphoenolpyruvate of a low M_r heat-stable protein, **HPr**. *Compare* **enzyme II** (def. 1).

enzyme II 1 EC 2.7.1.69; enzyme II of the **phosphotransferase** system; protein-N^{π}-phosphohistidine sugar phosphotransferase: *systematic name*: protein-N^{ϵ}-phosphohistidine:sugar N^{π}-phosphotransferase. Any of a group of related membrane-bound bacterial enzymes, part of the system for the transport of hexoses across the cell membrane, that catalyse the phosphorylation of hexoses by phospho-HPr (*see* **enzyme I**). Enzyme II is responsible for the specificity of the transport process with respect to the sugar. 2 *a former name for* **acyl-carrier protein**.

enzyme-activated irreversible inhibitor *an alternative name for* **suicide inhibitor**.

enzyme activation the generation of a catalytically active enzyme from an inactive or poorly active form or from a biosynthetic precursor (proenzyme). Activation may be by enzymic or chemical covalent modification or by addition of a specific activator.

enzyme adaptation *see* **inducible enzyme**.

enzyme cascade *see* **cascade sequence**.

enzyme classification the systematic arrangement and naming of enzymes by the **Enzyme Commission**. Each enzyme is denoted by the abbreviation EC followed by a set of four numbers separated by stops. The first number denotes one of the six main divisions: EC 1, oxidoreductases; EC 2, transferases; EC 3, hydrolases; EC 4, lyases; EC 5, isomerases; and EC 6, ligases. The second number denotes the subclass, the third number denotes the sub-subclass; the fourth number is the serial number of the particular enzyme. The most recent edition of the classification, *Enzyme Nomenclature 1992* (IUB/Academic Press: San Diego, London), lists 3196 enzymes.

enzyme cluster *or (sometimes)* **multienzyme cluster** 1 any physiologically significant system of two or more enzymes in physical (i.e. noncovalent) association. The term embraces any **enzyme complex**, **multienzyme complex**, or membrane-bound enzyme array. Clustered enzymes usually display different kinetic and/or regulatory features from their unassociated counterparts; they may be encoded in a cluster-gene. *See also* **multienzyme system**. 2 *an alternative term for* **cluster** (def. 2).

Enzyme Commission *or* **the International Commission on Enzymes**: a body established in 1956 by the International Union of Biochemistry (IUB) to consider the classification and nomenclature of enzymes and coenzymes, their units of activity and standard methods of assay, together with the symbols used in the description of enzyme kinetics. The Commission, which worked closely with the Biological Chemistry Nomenclature Commission of the International Union of Pure and Applied Chemistry (IUPAC), was dissolved in 1961 and its work has been carried on in turn by the Standing Committee on Enzymes of IUB, by the IUPAC/IUB Joint Commission on Biochemical Nomenclature (JCBN), by an Expert Committee on Enzymes, and most recently by the Nomenclature Committees of IUB (NC-IUB) or the International Union of Biochemistry and Molecular Biology (NC-IUBMB). *See also* **enzyme classification**.

enzyme complex an operational term for any structural and functional entity composed of a number of dissociable enzymes that catalyse a sequence of closely related chemical reactions. *Compare* **multienzyme**.

enzyme detergent any detergent preparation incorporating an enzyme to assist its cleansing action. The enzymes used in such detergents are usually proteinases of high thermal and alkaline stability, e.g. **Alcalase**.

enzyme differentiation the process whereby, during the development of an organism, each tissue acquires its own characteristic quantitative pattern of enzymes, which underlies the physiological functions and morphological features of the tissue. From a fairly uniform enzymic make-up in the cells of the early embryo, the enzyme patterns of different tissues become progressively more differentiated as development into the mature organism proceeds.

enzyme electrode any electrode, incorporating an enzyme into its structure, that responds to the concentration of one of the substrates or products of the reaction catalysed by the enzyme. The enzyme is trapped within a gel matrix surrounding the electrode or is kept in contact with the electrode by a semipermeable membrane.

enzyme engineering *or* **enzyme technology** the branch of biomolecular engineering concerned with processes designed to produce, isolate, purify, and immobilize enzymes and to use them for the catalysis of specific chemical reactions.

enzyme immunoassay *or* **enzymoimmunoassay** *abbr.*: EIA; any **immunoassay** in which an enzyme-catalysed reaction is used as the indicator. *See also* **heterogeneous immunoassay**, **homogeneous immunoassay**.

enzyme induction the synthesis of an enzyme in a cell or organism at a markedly increased rate in response to the presence of an **inducer**. The inducer is thought to combine with a **repressor** thereby preventing the latter from blocking an **operator**, which controls the translation of the structural gene for the enzyme.

enzyme-inhibition immunoassay a variation of **enzyme immunoassay** in which the inhibition of an enzyme-catalysed reaction is used as the indicator.

enzyme labelling a method used to detect or locate (and sometimes estimate) an antigen in, e.g., a tissue section. The section is exposed to a complementary antibody that has been covalently linked to an enzyme; the antibody binds to the antigen, and its location (and its amount) is determined by an assay dependent on the catalytic activity of the linked enzyme. *See also* **enzyme immunoassay**.

enzyme-linked immunosorbent assay *abbr.*: ELISA; a form of quantitative **immunoassay** based on the use of antibodies (or antigens) that are linked to an insoluble carrier surface, which is then used to 'capture' the relevant antigen (or antibody) in the test solution. The antigen–antibody complex is then detected by measuring the activity of an appropriate enzyme that had previously been covalently attached to the antigen (or antibody).

enzyme membrane any (semipermeable) membrane to which an enzyme has been covalently bound. Such membranes are useful in constructing **enzyme electrodes**.

enzyme-multiplied immunoassay technique *abbr. and proprietary name*: EMIT; *an alternative name for* **homogeneous enzyme immunoassay**.

enzyme-paper graft an enzyme immobilized on (filter) paper, which is frequently impregnated with indicators. It is useful for making analytical devices.

enzyme reactor a device for using immobilized enzymes or enzyme systems for synthetic or other processing reactions, especially on an industrial scale.

enzyme recruitment the exploitation of substrate-ambiguous enzymes or transport proteins in the evolution of new biochemical pathways.

enzyme repression inhibition of the formation of an enzyme by a compound formed in or taken in by a cell or organism.

enzyme specificity *see* **specificity**.

enzyme–substrate complex *abbr.*: ES; the stoichiometric complex of an enzyme molecule and a substrate molecule bound at the enzyme's active site.

enzyme technology *an alternative term for* **enzyme engineering**.

enzyme unit *symbol*: U; *abbr.*: EU; an obsolete unit of activity of enzymes, defined as the amount of enzyme that will catalyse the transformation of one micromole of the substrate per minute under standard conditions. It has now been superseded by the **katal**.

enzymic *or* **enzymatic** by, of, involving, or relating to an enzyme or enzymes; catalysed by an enzyme or by enzymes. — **enzymically** *or* **enzymatically** *adv.*

enzymic activity the rate of reaction of substrate that may be attributed to catalysis by an enzyme. The concept is now obsolete, having been superseded by **catalytic activity**. The unit of

EXHIBIT

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Human Immunodeficiency Virus Type 1 Vpr Protein Is Incorporated into the Virion in Significantly Smaller Amounts than Gag and Is Phosphorylated in Infected Cells

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Viral protein R (Vpr) of human immunodeficiency virus type 1 (HIV-1) is a small accessory protein involved in the nuclear import of viral DNA and the growth arrest of host cells. Several studies have demonstrated that a significant amount of Vpr is incorporated into the virus particle via interaction with the p6 domain of Gag, and it is generally assumed that Vpr is packaged in equimolar ratio to Gag. We have quantitated the relative amount of Vpr in purified virions following [^{35}S]cysteine labeling of infected MT-4 cells, as well as by quantitative immunoblotting and found that Vpr is present in a molar ratio of approximately 1:7 compared to capsid. Analysis of isolated core particles showed that Vpr is associated with the mature viral core, despite quantitative loss of p6 from core preparations. Metabolic labeling of infected cells with ortho[^{32}P]phosphate revealed that a small fraction of Vpr is phosphorylated in virions and infected cells.

Viral protein R (Vpr), a polypeptide of 96 amino acids, is the major virion-associated accessory protein of human immunodeficiency virus type 1 (HIV-1). Studies in tissue culture revealed two main biological functions of Vpr. First, it prevents host cell proliferation by arresting cells in the G_2 phase of the cell cycle. This effect has been related to induction of cell death as well as to increased viral gene expression, but its exact role for viral replication is unclear (15, 17, 20, 30, 33, 34, 36, 45). An independent function of Vpr is to promote the transport of the viral genome into the nucleus, thereby contributing to the ability of HIV-1 to infect nondividing cells (10, 18, 31, 32, 38). Several previous studies have demonstrated that HIV-1 Vpr as well as the related Vpx proteins of HIV-2 and simian immunodeficiency virus (SIV) are selectively packaged into virus particles (9, 46, 47), supporting a role in the early phase of virus replication. Incorporation into the virion occurs via specific interaction with the p6 domain of the Gag polyprotein (1, 3, 23, 24, 27, 29, 35), and this interaction can be exploited to target proteins in *trans* into the HIV particle via fusion to Vpr (43). Although the immunoprecipitation experiments which defined Vpr as a virion component did not allow exact quantitation, it is generally assumed that Vpr is present in equimolar amounts to Gag in HIV-1 particles (8).

The study presented here was aimed at a more detailed characterization of the virion-associated Vpr protein. First, we applied two different techniques to determine the amount of Vpr incorporated into virus particles. One approach involved the production of metabolically labeled virus particles from MT-4 cells. Cells were infected with HIV-1 strain NL4-3 by coculture as described previously (42). At 24 h postinfection, cells were incubated for 2 h in cysteine-free medium. Following this starvation period, steady-state labeling was performed by addition of [^{35}S]cysteine (20 μCi/ml) to cysteine-free culture medium containing dialyzed fetal calf serum. Tissue culture supernatant was harvested following an additional 18 to 36 h of incubation. To directly visualize the major viral proteins without prior immunoprecipitation, particles were pelleted from the clarified supernatant through a 20% (wt/wt) sucrose cushion and further purified by banding on an OptiPrep velocity gradient, adapted from a recently published protocol (12). Gradient fractions containing viral particles were identified as a visible band and were collected and concentrated by centrifugation. Virion-associated proteins were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

A characteristic pattern of radiolabeled protein bands was observed, and immunoprecipitation with specific polyclonal antisera was carried out to confirm the identity of particle-associated proteins (Fig. 1 and data not shown). As apparent in Fig. 1, 2A, and 4, the band corresponding to Vpr was clearly detectable in the purified virus preparations. Since virus lysates were highly pure as judged by silver staining of SDS-gels and [^{35}S]cysteine-labeled proteins were easily identifiable without prior immunoprecipitation (Fig. 2A), the radioactivity contained in the Vpr, capsid (CA), and matrix (MA) bands could be directly quantitated by phosphorimage analysis. Virus material from three independent labeling experiments was used for evaluation. After correcting for the number of cysteines present in the proteins, amounts of MA, Vpr, and integrase (IN) in virus preparations were calculated relative to the CA protein (arbitrarily set as 100) (Fig. 2B). As expected, amounts of CA and MA calculated were nearly identical, whereas the amount of the *pol*-derived IN was 50-fold lower. In contrast, we found a ratio of Vpr to CA of only about 1 to 7. If one assumes an average number of 1,800 CA molecules per virion, as has been determined for retroviral particles by scanning transmission electron microscopy (39), one particle contains approximately 275 molecules of Vpr. These calculations are based on the assumption that translation efficiency and turnover of Vpr and CA are comparable, resulting in comparable relative ^{35}S incorporation into both proteins. Since we cannot formally exclude differences in this respect, the result obtained from the labeling experiment was confirmed by an independent ap-

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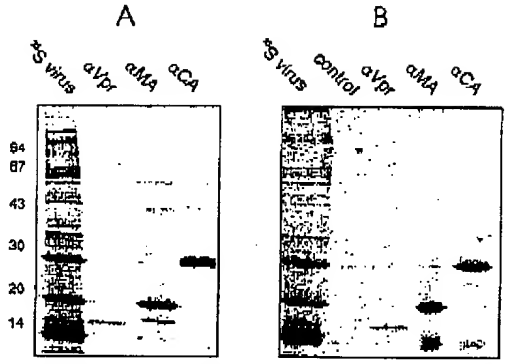

FIG. 1. Identification of [^{35}S]cysteine-labeled virion proteins by immunoprecipitation. A total of 3×10^6 infected cells (A) or purified virus equivalent to 2.4 ml of tissue culture supernatant (B) were lysed in 750 μl of radioimmunoprecipitation assay buffer containing 2 mM Pefabloc, 10 μM E64, and 1 μM pepstatin. Extracts were subjected to immunoprecipitation according to standard procedures (16), using polyclonal rabbit antisera directed against the indicated HIV proteins or an unrelated antibody (control) bound to protein A-agarose (Roche). Immunoprecipitates were separated by SDS-PAGE (17.5% acrylamide; acrylamide/bisacrylamide, 200:1) and visualized by phosphorimage analysis using a Fuji BAS2000 instrument. As a reference, an aliquot of purified labeled virus was also loaded directly onto the gel (lane ^{35}S virus). Positions of marker proteins and their molecular masses in kilodaltons are indicated at the left.

proach using quantitative immunoblotting. Unlabeled virus was prepared from infected MT-4 cells and purified as described above. Virion-associated Vpr and CA proteins were detected by immunoblotting with specific polyclonal antisera (Fig. 2C). For detection of Vpr, we used a polyclonal rabbit antiserum prepared against the full-length protein. Serial dilutions of recombinant HIV-1 CA and synthetic full-length Vpr peptide were analyzed in parallel. Densitometric evaluation and comparison of reactivities of the virion-derived proteins with those of the standards of known concentration yielded approximate amounts of 1.65 μg of CA and 0.11 μg of Vpr in 2 μl of virus sample, corresponding again to a molar ratio of CA to Vpr of about 7 to 1. The observation that HIV-1 particles contain Vpr in significantly lower amount than Gag is in contrast to the general assumption that Vpr and Gag are packaged in equimolar amounts in the virion; however, to our knowledge the data presented here are the first quantitative analysis of Vpr incorporation into HIV-1 particles released from infected cells not involving transcomplementation with Vpr. Since Vpr is incorporated into the particles via direct interaction with the p6 domain of Gag, packaging of a stoichiometric amount should theoretically be possible. However, the intracellular concentration of Vpr available during virus assembly may be limited due to Vpr turnover or binding to cellular factors. Although the interaction between $Pr55^{Gag}$ and Vpr is strong enough to be measured by in vitro assays and has been reported to be stable against high NaCl concentrations (3, 35), the affinity between the two proteins has not been quantitated. In the case of HIV-2, it has been shown that the relatively low amount of Vpr incorporated into virions is related to the short (<90-min) half-life of intracellular Vpr and can be increased by overexpression of the protein in *trans*, while the related Vpx protein is much more stable (half-life of >36 h) and is incorporated in comparable amounts to Gag (22). It appears less likely that a similar effect is responsible for the less than stoichiometric incorporation of HIV-1 Vpr, since its half-life in MT-4 cells has been reported to be considerably

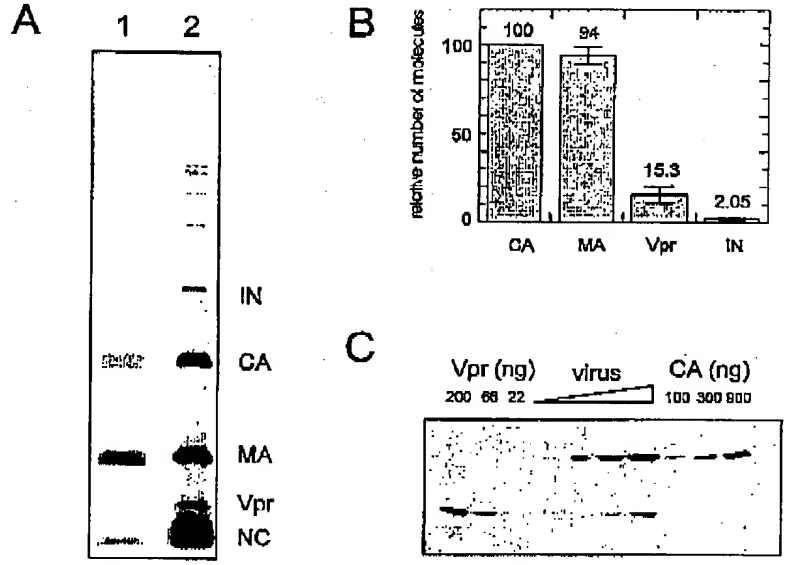

FIG. 2. Determination of the relative amounts of Vpr and CA in virus lysates. (A) Aliquot of a purified ^{35}S-labeled virus sample used for quantitative analysis. The sample was analyzed by SDS-PAGE followed by silver staining as described by Heukeshoven and Dernick (19) (lane 1) as well as phosphorimage detection (lane 2). (B) Radioactivity in bands corresponding to Vpr, MA, CA, and IN contained in virus samples as determined by phosphorimage analysis. After normalizing for the number of cysteines present, average numbers of molecules were calculated relative to the intensity of the CA band in the same lane, which was arbitrarily set at 100%. Data shown represent the mean relative value calculated for each protein. (C) Relative amounts of virion-associated CA and Vpr as determined by immunoblotting. Various amounts of purified virus were separated by SDS-PAGE in parallel to serial dilutions of recombinant CA protein or synthetic Vpr protein of known concentration. Proteins were detected by immunoblotting using rabbit antisera directed against CA or Vpr followed by enhanced chemiluminescence staining. Band intensity was measured by densitometry using a Desaga CD50 instrument.

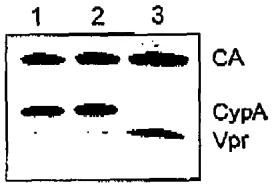

FIG. 3. Immunoblot analysis of purified HIV-1 virions and isolated core particles. Mature core particles were prepared as described by Welker et al. (42). Virus particles pelleted through a sucrose cushion (lane 1) and further purified by banding in an OptiPrep gradient (lane 2) as well as a preparation of core particles from OptiPrep gradient-purified virions (lane 3) were separated by SDS-PAGE. Similar amounts with respect to CA were loaded in each lane. Virion-associated proteins were identified by immunoblotting using a mixture of antisera against the indicated proteins and enhanced chemiluminescence staining. CypA, cyclophilin A.

longer than 7 h (44). Nevertheless, the intracellular Vpr concentration seems to play an important role for incorporation efficiency, since in cells transfected with HIV-1 DNA the amount of virion-associated Vpr-derived protein is increased upon overexpression of Vpr fusion proteins in *trans* (43), suggesting that the potential interaction sites on the Gag protein are not saturated by Vpr expressed from the proviral DNA.

Contrasting results have been reported concerning the subviral localization of HIV-1 Vpr and the related Vpx protein of HIV-2 and SIV. Whereas immunoelectron microscopy studies suggested that HIV-1 Vpr is located mainly beneath the virion membrane (40) and Vpx from SIV_{mac} was also detected outside the virus core (26), HIV-2 Vpx was found associated with mature cores (21). To biochemically analyze subviral Vpr localization, we adapted a method developed in our lab for preparation of intact HIV core particles by detergent stripping (42), but used gradient purified virus as starting material. Comparative immunoblot analysis of virions and isolated core particles (Fig. 3) revealed that Vpr was significantly enriched in the core preparations, whereas p6, as well as other HIV-1 structural proteins (MA) and the virion-associated cellular protein cyclophilin A, were quantitatively removed by detergent treatment (Fig. 3 and reference 42). Segregation of Vpr and p6 was surprising, because the p6 domain of Gag carries the binding site for Vpr and is presumed to recruit Vpr into the virion. Conceivably, cleaved p6 has a reduced affinity toward Vpr, resulting in dissociation of the complex upon maturation. This possibility is supported by the finding that p6, in contrast to $Pr55^{Gag}$, does not display interaction with Vpr in a yeast two-hybrid analysis (35). Vpr may be retained to the core by being associated with the complex of nucleocapsid protein (NC) and the viral genomic RNA. Consistent with this hypothesis, an affinity of Vpr toward NC (11, 25, 35) as well as toward nucleic acid (48) has been reported. In any case, HIV-1 Vpr is clearly a core-associated protein, which is likely to be important for its functions in early virus replication.

Posttranslational modifications might serve to regulate the diverse functions of Vpr. Since modification of viral proteins by kinases is known as an important way to regulate viral replication, we were interested in potential phosphorylation of Vpr. Intracellular phosphorylation of several HIV-1 proteins (MA, CA, Vpu, Vif, and Nef) has been reported and, in the case of MA and Vpu, has been implicated in the regulation of differential activities of these proteins (8). To determine whether phosphorylation of Vpr occurs in infected cells, MT-4 cells were metabolically labeled with 0.5 mCi of ortho[^{32}P]phosphate per ml at 18 to 24 h postinfection with HIV-1 strain NL4-3. Twelve hours later, virus was harvested and purified by banding in a velocity gradient as described above. Virus preparations as well as infected cells were lysed in standard radioimmunoprecipitation assay buffer (16) containing 1 mM sodium orthovanadate, 2 mM Pefabloc, 10 μM E64, and 1 μM pepstatin, and lysates were subjected to immunoprecipitation with antisera against various HIV proteins. From lysates of infected cells, antiserum against Vpr precipitated a radiolabeled protein with the expected apparent molecular weight (Fig. 4A), demonstrating that there is indeed a phosphorylated form of Vpr. In the same series of experiments we also detected phosphorylated forms of MA and CA, whose occurrence is well documented in previous reports (6, 7, 14, 28, 37). Unspecific cross-reactivity of the sera was excluded by parallel experiments using lysates of equally labeled uninfected cells, where none of the bands shown in Fig. 4 were detected (not shown). A radiolabeled Vpr band was also observed when purified virus lysate was used for immunoprecipitation (Fig. 4B), indicating that a phosphorylated form of Vpr (pVpr) is associated with virus particles.

To determine the relative amount of pVpr in virus particles, we performed denaturing two-dimensional gel electrophoresis of unlabeled and ^{32}P-labeled virus samples (Fig. 5). Several forms of Vpr with different isoelectric mobilities were detected in the unlabeled virus preparation by immunoblotting (Fig. 5A). Using several independent virus preparations, we consistently observed two major isomeric forms of Vpr with apparent isoelectric points (IEP) of approximately 7.3 and 6.8 and with minor spots focusing at pH 6.3 and 8.0, respectively. Only a single spot with the apparent molecular weight of Vpr, focusing at pH 6.3, was detected in analyses of ^{32}P-labeled virus from two independent preparations (Fig. 5B). We conclude that pVpr corresponds to the minor form of Vpr indicated by an arrow in Fig. 5A. The difference between the nonphosphorylated Vpr forms leading to different apparent IEPs may be due to proteolytic removal of charged amino acids or other minor modifications like the change of an amide side group to a carboxy group. Analyses of [^{35}S]cysteine-labeled virus (not shown) also revealed the Vpr isoform focusing at pH 6.3, and phosphorimage analyses allowed us to estimate that pVpr rep-

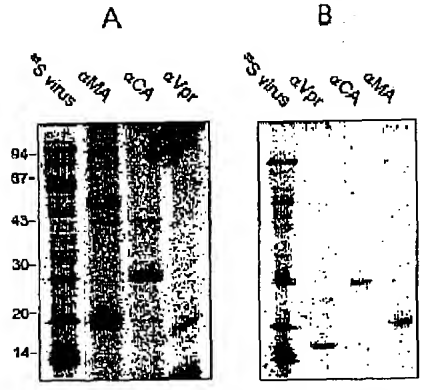

FIG. 4. Immunoprecipitation of ^{32}P-labeled HIV-1 proteins. Lysate from 3.5×10^6 infected cells metabolically labeled with ortho[^{32}P]phosphate (A) or purified virus equivalent to 18 ml (Vpr) or 2.25 ml (MA and CA) of tissue culture supernatant (B) was subjected to immunoprecipitation as in Fig. 1, using the indicated antisera. Immunoprecipitates were separated by SDS-PAGE and visualized by phosphorimage analysis. As a reference, an aliquot of [^{35}S]cysteine-labeled virus lysate was separated on the same gel (lane ^{35}S virus). Positions of marker proteins and their molecular masses in kilodaltons are indicated at the left.

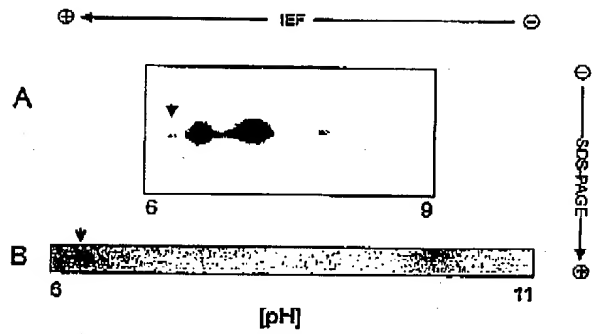

FIG. 5. Analysis of virion-associated Vpr by two-dimensional gel electrophoresis. (A) Unlabeled virus released from infected MT-4 cells was gradient purified as described for the labeled virus preparations. A sample corresponding to 15 μg of CA was applied to an Immobiline DryStrip 3-10L (Amersham Pharmacia) and subjected to isoelectric focusing (IEF) under denaturing conditions (8 M urea) according to the manufacturer's instructions, using an IGphor unit. Separation in the second dimension was performed by SDS-PAGE; subsequently, Vpr was detected by immunoblotting using antiserum directed against synthetic Vpr. (B) Gradient-purified ^{32}P-labeled virus equivalent to 20 ml of tissue culture supernatant was separated by IEF (Immobiline DryStrip 6-11L) followed by SDS-PAGE. Radiolabeled protein was detected by phosphorimage analysis. Arrows indicate a single phosphorylated protein spot of the apparent molecular weight of Vpr, corresponding to an IEF of 6.3 (B) and an immunoreactive spot corresponding to the same IEF (A).

resents approximately 5% of total virion-associated Vpr. This result is supported by comparison with the relative labeling intensity of phosphorylated CA. Parallel two-dimensional analyses of labeled and unlabeled virion-associated CA (not shown) revealed a single phosphorylated form, representing approximately 5% of virion-associated CA. In the experiment shown in Fig. 4B, the labeling intensities of the immunoprecipitated Vpr, MA, and CA bands were almost identical. Since in this case eight times more virus lysate was used for immunoprecipitation with anti-Vpr serum than for precipitation with anti-MA or anti-CA serum, correcting for the different relative amounts of these proteins in the virion, we estimate that virus-associated Vpr is phosphorylated to a similar extent as CA. Virion-associated MA was also found to be phosphorylated to a similar degree.

Vpr of NL4-3 contains 11 residues (four Ser, four Thr, and three Tyr) which theoretically could be phosphorylated. We have not yet mapped the modified residue(s), but one might consider Ser79 as a candidate phosphorylation site, based on sequence- and structure-dependent computer prediction according to Blom et al. (4) together with the absolute conservation of this residue in HIV-1 Vpr. It is tempting to speculate that Vpr phosphorylation plays a role in regulating the multiple functions of the protein in virus replication. Whereas in the cases of HIV-2, SIV_{sm}, and SIV_{mac} two independent proteins, Vpr and Vpx, are required for the nuclear import of the viral genome and the induction of host cell growth arrest, in the case of HIV-1 a single protein is responsible for both functions. Vpr also displays numerous other activities in tissue culture, like transcriptional activation, cell killing, or induction of cell differentiation. Consistent with that, the association of Vpr with a number of viral and cellular factors has been reported (2, 5, 11, 13, 25, 32, 38, 41). Vpr phosphorylation and dephosphorylation may be used to modulate these interactions throughout the viral replication cycle. Further studies are aimed at identification of the modified amino acid residue(s) as a prerequisite for testing this hypothesis.

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